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Chimeric Galanin Analogs That Function as Antagonists in the CNS are Full Agonists in Gastrointestinal Smooth Muscle

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ABSTRACT

Galanin has numerous effects on gastrointestinal smooth muscle. However, because of the lack of specific inhibitors, it is not known which are physiological and which are pharmacological. This study investigates the ability of two chimeric galanin analogs, [#1-galantide = (M-15) = [galanin (1-13)-substance P(5–11)] and #2-M-35[galanin(1–13)bradykinin (2–9)], which were recently reported to function as galanin-receptor antagonists in the CNS, to interact with galanin receptors on rat jejunal muscle strips or dispersed smooth muscle cells from guinea pig stomach. In both systems each chimeric analog had agonist activity and was as efficacious as galanin. Cross-desensitization experiments demonstrated that in the jejunal muscle strips, both chimeric analogs were causing muscle contraction by interacting with the galanin receptor. In dispersed smooth muscle cells, galanin, as well as each chimeric analog, caused muscle relaxation, whereas substance P and bradykinin both caused muscle contraction. Each chimeric analog was equipotent to galanin in inhibiting binding of ^{125}I-galanin, and there was close agreement between their abilities to occupy the galanin receptor and cause relaxation. Each chimeric analog also activated adenylate cyclase and increased cAMP characteristic of relaxants. These studies demonstrate these chimeric analogs will not be useful for defining the physiological role of galanin in altering gastrointestinal motility, because they function as full galanin-receptor agonists instead of as galanin-receptor antagonists.

Galanin is a 29-amino-acid peptide originally isolated from porcine intestine in 1983 (Tatemoto *et al.*, 1983). Subsequently, galanin-like immunoreactivity has been shown to be widely distributed in the central nervous system (Rokaeus *et al.*, 1984; Rokaeus, 1987) as well as in peripheral tissues, where, in such tissues as the gastrointestinal tract, it is present in both the myenteric and Meissner's plexi and in neural elements in the smooth muscle layers (Ekblad *et al.*, 1985; Melander *et al.*, 1985). Specific high-affinity galanin receptors have also been described both in the central nervous system and in peripheral tissues, including gastrointestinal smooth muscle (King *et al.*, 1989; Fisone *et al.*, 1989; Gu *et al.*, 1992b; Rossowski *et al.*, 1990).

Galanin has numerous effects both in the central nervous system and in peripheral tissues (Rokaeus, 1987; Bartfai *et al.*, 1992; Rattan, 1991). In the central nervous system, it has been shown to coexist with other classical neurotransmitters or peptides that function as neurotransmitters and in some cases to inhibit their release. For example, it inhibits the release of acetylcholine in the ventral hypocampus (Bartfai *et al.*, 1992; Fisone *et al.*, 1987). Galanin has been found to decrease firing of noradrenergic neurons in the locus caeruleus (Seutin *et al.*, 1989; Bartfai *et al.*, 1992), and it has been proposed as a major determinant of nonadrenergic tonus in the central nervous system (Bartfai *et al.*, 1992). It has also been proposed that galanin has an important inhibitory role in sensory transmission in the spinal cord (Rokaeus, 1987; Bartfai *et al.*, 1992). In peripheral tissues galanin has endocrine effects, such as inhibition of glucose-induced insulin release, as well as effects on gastrointestinal motility (Lindskog and Ahren, 1987; Rokaeus, 1987; Rattan, 1991). In various species it causes gastrointestinal smooth muscle contraction, relaxation or modulations of the effects of other peptides or neurotransmitters on gastrointestinal smooth muscle contractility; therefore, it may be important in regulating gastrointestinal motility (Ekblad *et al.*, 1985; Rattan, 1991; Bartfai *et al.*, 1992).

At present, it is not clear which of these effects of galanin on gastrointestinal motility are physiological. Recently two groups of novel chimeric galanin analogs have been described that are reported to function as potent galanin-receptor antagonists (Bartfai *et al.*, 1991; Wiesenfeld-Hallin *et al.*, 1992; Bartfai *et al.*, 1992), thus raising the possibility they can be used to define

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ABBREVIATIONS: Galantide, [galanin(1–13)-substance P(5–11)amide]; M-35, [galanin(1–13)-bradykinin(2–9)amide]; IBMX, 3-isobutyl-1-methylxanthine.

galanin's physiological role in various processes. One analog, galantide (M-15) [galanin(1–13)substance P(5–11)] has been shown to have an affinity of 0.1 nM for galanin receptors in the ventral hippocampus, to inhibit galanin's effect on the flexor reflex and to cause inhibition of glucose-induced insulin release and inhibition of acetylcholine release (Bartfai *et al.*, 1991; Lindskog *et al.*, 1992). The other analog, M-35, is reported to have an affinity constant of 0.3 nM for galanin receptors in rat dorsal spinal cord and to inhibit galanin's effect on the flexor reflex (Wiesenfeld-Hallin *et al.*, 1992). In the process of assessing the possible usefulness of these chimeric analogs for defining the physiological role of galanin in regulating gastrointestinal motility, we found that each of these chimeric analogs had agonist activity. In this report we investigate the basis for the agonist activity of two different gastrointestinal smooth muscle preparations and demonstrate that they function as full receptor agonists at the galanin receptor.

Materials and Methods

Adult male Charles River rats weighing 200 to 300 g were used for the experiments assessing jejunal muscle contractility. Rats were housed under standard conditions and kept in an artificial 12-hr light cycle and allowed free access to rat Purina chow and water. Rats were sacrificed and fragments of the jejunum 10 to 15 mm long were immediately removed, cleaned, and placed into Krebs-Henseleit buffer containing 25 mM sodium bicarbonate and 2.5 mM $CaCl_2$, pH 7.4, oxygenated with a mixture of O_2 (95%) and CO_2 (5%) at 37°C.

Male guinea pigs (150–300 g) obtained from the Small Animal Section, Veterinary Resources Branch, National Institutes of Health, were used in the experiments assessing the contractility of isolated gastric smooth muscle cells. Drugs and chemicals were obtained from the sources indicated: HEPES (Boehringer Mannheim Biochemical, Indianapolis, IN), collagenase (type CLS 2) (Worthington Biochemical, Freehold, NJ), carbamylcholine chloride (carbachol), bacitracin, leupeptin, soybean trypsin inhibitor and IBMX, Krebs-Henseleit buffer mixture, acetylcholine chloride, guanethidine sulfate, amastatin hydrochloride and phosphoramidon [N-(-Rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp] sodium salt (Sigma Chemical, St. Louis, MO), Eagle's basal amino acid medium (100-times concentrated) (GIBCO, Grand Island, NY), essential vitamin mixture (100-times concentrated) (Microbiological Associates, Bethesda, MD), glutamine (Research Plus Laboratories, Bayonne, NJ), bovine plasma albumin (fragment V) (Miles Laboratories, Elkhart, IN), chymostatin, bestatin, vasoactive intestinal peptide (VIP) and porcine galanin (Peninsula Laboratories, Belmont, CA), ^{125}I-galanin (porcine), ^{125}I-succinyl cAMP tyrosine methylester and cAMP antiserum (preconjugated to a second antibody) (New England Nuclear, Boston, MA). Galanin-(1–15), rat galanin-(1–29), galantide (also called M-15 in Bartfai *et al.*, 1991), M-35, substance P, spantide and bradykinin were synthesized by standard solid phase methods. After hydrogen fluoride cleavage, peptides were purified using multiple Sephadex G-25 and preparative high-performance reversed phase liquid chromatography steps to greater than 95% purity, as determined by analytical high-performance liquid chromatography and amino acid analyses of acid hydrolysates.

Effect of agents on rat jejunal muscle contractility. Longitudinal smooth muscle strips of the jejunum were attached to a transducer (Narco Bio-Systems, Myograph Transducer, F-60) and placed into a siliconized 20-ml organ bath (Harvard Instruments) containing Krebs-Henseleit buffer maintained at 37°C. Isometric contractions were recorded with a Narcotrace 40 (Narco Bio-Systems, Houston, TX). Baseline tension was set at 0.5 g, and the rat jejunum longitudinal smooth muscle strips were equilibrated for 40 to 60 min, the buffer being changed every 20 min. All experiments began with testing the standard, acetylcholine chloride (0.5 μM), and only tissues responding properly were used for further studies. The magnitude of contraction of the rat jejunal longitudinal smooth muscle strips induced by 0.5 μM acetylcholine was considered 100%, and any contractions induced by tested peptides were expressed in percentages. In order to eliminate possible involvement of cholinergic and adrenergic components in the test responses, the experiments were carried out in the presence of atropine sulfate (1 μM) and guanethidine (3 μM). To prevent possible proteolytic degradation, the aminopeptidase inhibitor amastatin (10 μM) and the neutral endopeptidase inhibitor phosphoramidon (1 μM) were included in the incubation buffer. In the control experiments, it was determined that these inhibitors at these concentrations did not affect the contraction of rat jejunal longitudinal smooth muscle strip. Concentration-response curves of galantide, M-35 [galanin-(1–13)-bradykinin-(2–9)-amide] and galanin-(1–15) were obtained by the single addition of each peptide at an interval of at least 20 min, with intermittent washings of the rat jejunal longitudinal smooth muscle strips. The response of rat jejunal longitudinal smooth muscle strip to each of the tested peptides was determined by measuring the change in amplitude. A maximum of four concentrations of peptide were tested with a single jejunal preparation. Each experiment was repeated using 4 to 6 separate rat jejunal longitudinal smooth muscle strips obtained from different animals. All EC_{50} values were calculated by means of nonlinear regression analysis of the concentration-response curves using SigmaPlot computer software.

Dispersed gastric smooth muscle cell preparation. To investigate directly the ability of galanin and the related chimeric analogs to interact with galanin receptors on smooth muscle cells, we prepared isolated dispersed smooth muscle cells from the guinea pig stomach, which have recently been shown to have high-affinity galanin receptors whose occupation results in the activation of adenylate cyclase and smooth muscle relaxation (Gu *et al.*, 1992b). Dispersed gastric smooth muscle cells were prepared as described previously (Bitar and Makhlouf, 1982a). Briefly, the mucosa was removed from each tissue by blunt dissection, and the muscle layer was cut into small pieces. The pieces were incubated for two successive 45-min periods at 31°C in 20 ml of a standard incubation solution containing 150 units/ml collagenase. After the second incubation period, the partially digested muscle pieces were washed with enzyme-free solution to allow the cells to disperse spontaneously. The cells were harvested by filtration through 500-μm Nitex Mesh (Baxter, McGaw Park, IL).

Unless otherwise stated, the standard smooth muscle cell incubation solution contained the following (in mM): HEPES 24.5, NaCl 98, KCl 6, NaH_2PO_4 2.5, pyruvate 5, fumarate 5, glutamate 5, glucose 11.5, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and glutamine 2, as well as bovine plasma albumin (0.2% wt/vol), soybean trypsin inhibitor (0.01% wt/vol), basal amino acid medium (1% vol/vol) and essential vitamin mixture (1% vol/vol). The standard incubation solution was adjusted to pH 7.4 and equilibrated with 100% O_2. All incubations were performed at 31°C with 100% O_2 as the gas phase.

^{125}I-galanin binding to dispersed smooth muscle cells. Dispersed gastric smooth muscle cells (1×10^6 cells/ml) were incubated with 150 pM ^{125}I-galanin (porcine) for 45 min at 31°C alone or with the indicated concentrations of unlabeled ligands in standard smooth muscle cell incubation solution containing 0.1% bacitracin, 1 μg/ml leupeptin, 1 μg/ml chymostatin and 1 μg/ml bestatin. A 45-min incubation time was chosen because time-course studies demonstrated that maximal binding occurred at 45 min. Bound ligand was separated from unbound ligand by filtering 100 μl of cell suspension through 0.45-μm Millipore HA filter using a Millipore Model 1225 sampling manifold (Millipore, Bedford, MA). Each filter was washed with 5 ml of ice-cold standard incubation solution three times under reduced pressure and was then assayed for radioactivity using Auto-Gamma Scintillation Spectrometer (Packard, Downers' Grove, IL). All binding data in this paper, unless otherwise specified, represent saturable binding. Saturable binding was the binding measured with ^{125}I-galanin alone (total binding) minus the binding measured in the presence of 1 μM galanin (nonsaturable binding). In all experiments, nonsaturable binding was less than 10% of total binding.

Gastric smooth muscle contraction studies. The contractile

response was measured as described previously (Bitar and Makhlouf, 1982a). Briefly, after incubation of gastric smooth muscle cells (1.5×10^6/ml) in standard incubation solution containing the agents to be tested, the reaction was stopped after 30 sec by adding acrolein (final concentration, 1%). To determine the ability of an agent to cause relaxation, the ability of the peptide to inhibit stimulated contraction was assessed as described previously (Bitar and Makhlouf, 1982b). The cells were first incubated for 60 sec with the agents to be tested, and then the contractant was added. After 30 sec, the lengths of 50 cells were measured by micrometry.

Measurement of cyclic AMP in dispersed smooth muscle cells. Cellular cyclic AMP was determined by radioimmunoassay as described previously (Maton *et al.*, 1988; Zhang *et al.*, 1990). Dispersed muscle cells from two stomachs were suspended in 5 ml of incubation medium. Each incubation was 250 μl and contained 3×10^5 muscle cells. Measurements of cellular cyclic AMP were performed with 1 μM IBMX unless otherwise stated. Different dilutions of cell suspensions were used with different concentrations of peptides so that the amount of cyclic AMP measured remained on the linear portion of the standard curve of the cyclic AMP radioimmunoassay. The required different dilutions were determined in preliminary experiments.

Statistical analysis. The results presented are means ± S.E.M. of at least three separate experiments. The statistical analysis was performed by a paired Student's *t* test, and a P value < .05 was considered significant.

Results

Effect of galantide (M-15) on the contractility of jejunal longitudinal muscle. Galantide and galanin(1-15) (1 μM) caused contraction of jejunal longitudinal muscle strips (figs. 1-3). Galantide caused half-maximal stimulation at 104 ± 38 nM (fig. 3), whereas galanin-(1-15) (fig. 3) and rat galanin-(1-29) (not shown) caused half-maximal stimulation at 11.1 ± 5.2 nM and 2.5 ± 0.6 nM, respectively. Because of the contractant effect of galantide on rat jejunal longitudinal smooth muscle strips, we first investigated which part of this chimeric peptide

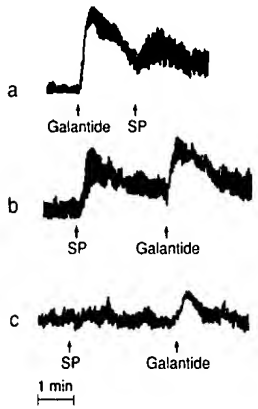

Fig. 1. Effect of galantide and substance P on the contractility of rat jejunal longitudinal muscle strips. In *panel a* galantide (1 μM) was added. After 2 min, substance P (SP) (1 μM) was added. In *panel b* is shown the reverse experiment with substance P (SP) added first, then galantide. In *panel c* rat jejunal longitudinal strip was desensitized by five subsequent cumulative additions of substance P (1 μM) at 4-min intervals. After the jejunal strip was no longer responsive to substance P, galantide (1 μM) was added.

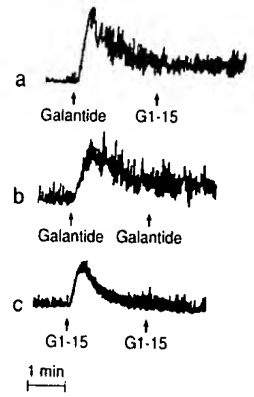

Fig. 2. Effect of galantide and galanin-(1-15) on the contractility of rat jejunal longitudinal muscle strips. In *panel a* the addition of galantide was followed in 2 min by the addition of galanin-(1-15) (1 μM). In *panel b* it was followed by a repeat addition of galantide (1 μM). In *panel c* galanin-(1-15) was added, followed in 2 min by a second addition of galanin-(1-15) (1 μM).

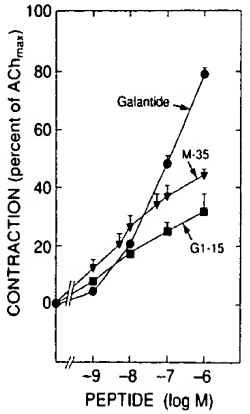

Fig. 3. Dose-response for contraction of rat jejunal longitudinal strips induced by galanin-(1-15) (■—■), galantide (●—●) and M-35 (▲—▲). Data are the means of four to seven separate experiments ± S.E.M. Data are expressed as the percent of maximal contraction caused by 0.5 μM acetylcholine.

is responsible for the jejunal longitudinal muscle contraction. Addition of the substance-P-receptor antagonist spantide at a concentration of 50 μM, which strongly inhibited the contractant effect of substance P (1 μM), did not affect contractory activity of galantide (data not shown). The addition of substance P (1 μM) 2 min before galantide or 1 to 4 min after galantide (10^{-6} M) resulted in the appearance of two separate contraction peaks, suggesting that galantide and substance P do not compete for the same receptor (fig. 1, a and b). To further prove this, jejunal strips were desensitized by the cumulative additions of substance P, and after the longitudinal smooth muscle strip was no longer responding to additional

doses of substance P, the addition of galantide (1 μM) continued to result in contraction (fig. 1c). These data suggest that the galantide contractant activity is not due to occupation of the substance P receptor and, therefore, is due to the presence of the galanin fragment of the galantide molecule. That galanin-(1-15) and galantide cause contraction through the same receptor is supported by the effects of galantide or galanin-(1-15) on subsequent contraction to galanin-(1-15) or galantide. The introduction of galantide into the incubation buffer, followed by the addition of galanin-(1-15) (1 μM), prevented the appearance of galanin-(1-15) (1 μM) contractant activity (fig. 2a). Similarly, neither a second dose of galantide (1 μM) after 2 min or a dose of galanin-(1-15) (1 μM) 2 min after galanin-(1-15) (1 μM) caused a second contraction (fig. 2, b and c).

Effect of M-35 on the contractility of rat jejunal longitudinal muscle. M-35 also caused contraction of rat jejunal longitudinal muscle (figs. 3 and 4) in a dose-dependent manner with a half-maximal effect at 7.2 ± 1.9 nM. After galanin-(1-15) (0.5 μM) induced contraction, the addition of 0.5 μM M-35 resulted in no additional contraction (fig. 4a), whereas the subsequent addition of bradykinin (0.5 μM) did result in a contraction (fig. 4a). On the other hand, the addition of bradykinin (1 μM) first did not block the subsequent contractile effect of M-35 (10^{-6} M) (fig. 4b). However, the initial addition of M-35 (1 μM) completely blocked the contractile effect of galanin-(1-15) (1 μM) (fig. 4c). Because the initial addition of galanin-(1-15), but not of bradykinin, blocked the contractile effect of M-35, these data suggest that M-35 stimulates contraction by interacting with the galanin receptor.

Effect of galanin or M-35 on contraction of smooth muscle cells. Dispersed gastric smooth muscle cells had a mean resting length of 110 ± 2 μm (table 1). Carbachol (30 nM) caused contraction and reduced the mean length to 87 ± 1 μm. Galanin, galantide or M-35 alone, at a concentration of 1 μM, did not affect the cell length of resting cells (table 1). However, all three of these peptides caused relaxation measured as functional antagonism of carbachol-induced contraction

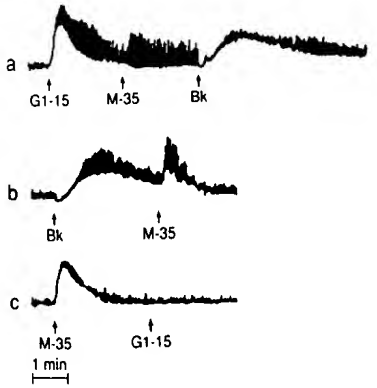

Fig. 4. Effect of galanin-(1-15) on M-35- and bradykinin-induced contraction of rat jejunal longitudinal muscle. In *panel a* galanin-(1-15) (0.5 μM) was first added, followed in 2 min by M-35 (0.5 μM) and in another 2 min by bradykinin (BK) (0.5 μM). In *panel b* bradykinin (BK) (1 μM) was added first, followed in 2 min by M-35 (1 μM). In *panel c* M-35 (1 μM) was added first, followed in 2 min by galanin-(1-15) (1 μM).

TABLE 1

Effects of galanin, galantide, M-35 and related peptides alone and in combination with carbachol on dispersed gastric smooth muscle cell length

Dispersed gastric smooth muscle cells were suspended in standard incubation solution and incubated at 31°C for 30 sec with the indicated peptides. In the combination studies, cells were incubated first with galanin, galantide or M-35, or with galantide and galanin or M-35 and galanin, for 60 sec. Then they were incubated with carbachol for another 30 sec. Values are means ± S.E.M. from at least three separate experiments. In each experiment, mean cell length was determined by measuring the length of 50 cells.

Additions	Cell Length (μm)
None	110 ± 2
Galanin (1 μM) alone	113 ± 3
Galantide (1 μM) alone	107 ± 4
M-35 (1 μM) alone	109 ± 1
Carbachol (20 nM) alone	87 ± 1*
Galanin (1 nM) and carbachol (30 nM)	98 ± 1**
Galanin (1 μM) and carbachol (30 nM)	108 ± 2**
Galantide (1 μM) and carbachol (30 nM)	106 ± 3**
M-35 (1 μM) and carbachol (30 nM)	108 ± 1**
Galantide (1 μM) and galanin (1 μM) and carbachol (30 nM)	108 ± 1
M-35 (1 μM) galanin (1 μM) and carbachol (30 nM)	107 ± 2
Substance P (1 μM)	94 ± 2*
Bradykinin (1 μM)	93 ± 2*

** = (P < .01) compared with carbachol alone. * = (P < .05) compared with control-cell length with no additions.

(table 1). To investigate whether galantide or M-35 antagonized galanin-induced relaxation, 1 μM galantide or 1 μM M-35 was added before galanin and carbachol. Neither galantide nor M-35 inhibited galanin-induced relaxation (table 1). The possibility existed that these peptides were altering carbachol-induced contractility by interacting with substance P or with bradykinin receptors. Because the substance P fragment(5-11) is in galantide and the bradykinin fragment(2-9) is in M-35, substance P and bradykinin were tested for their effect on gastric smooth muscle cell length. Both substance P and bradykinin caused cell contraction (table 1). These results suggest that galantide and M-35 do not cause cell relaxation by occupying substance P or bradykinin receptors. Rather, both of these chimeric peptides may be stimulating relaxation by occupying galanin receptors.

To determine their relative potencies for causing relaxation, the effects of various concentrations of the galanin-related peptides on carbachol-induced contraction were determined (fig. 5). Each of the chimeric analogs was approximately equipotent to galanin and caused a half-maximal effect at 2-7 nM (fig. 5; table 2). This suggests that if these chimeric analogs both alter contractility by occupying the galanin receptor, they should have an affinity for this receptor similar to that of galanin itself.

Abilities of galanin, galantide and M-35 to inhibit binding of ^{125}I-galanin to isolated gastric smooth muscle cells. A previous study has demonstrated that the direct interaction with galanin receptors on gastric smooth muscle cells can be investigated by using ^{125}I-galanin-binding studies (Gu *et al.*, 1992b). To determine the abilities of these peptides to interact directly with galanin receptors, their dose-response curves for inhibiting the binding of ^{125}I-galanin to galanin receptors on gastric smooth muscle cells was determined. When cells were incubated with ^{125}I-galanin and unlabeled galanin at 31°C for 45 min, detectible inhibition of the binding of ^{125}I-galanin by unlabeled galanin occurred at 0.1 nM, a half-maxi-

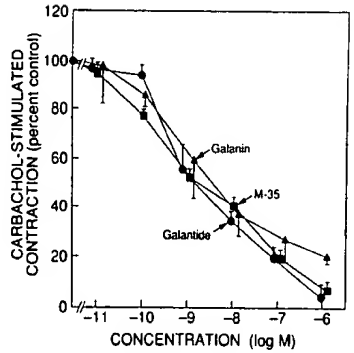

Fig. 5. Effect of various concentrations of galanin, galantide or M-35 on carbachol-induced contraction. Dispersed gastric smooth muscle cells were incubated with the galanin-related peptides at the indicated concentration at 31°C for 60 sec, followed by the addition of carbachol (30 nM); then they were incubated for another 30 sec. Results are expressed as the percent of the maximal contraction caused by carbachol (30 nM) alone. Results are means ± S.E.M. from at least three separate experiments. In each experiment, mean cell length was determined by measuring the lengths of 50 cells.

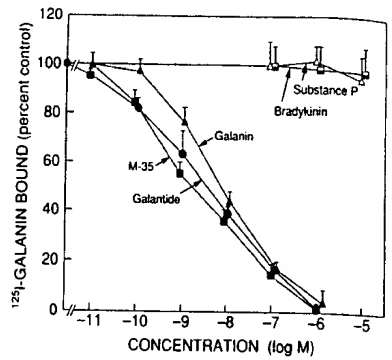

Fig. 6. Abilities of galanin, galantide, M-35 and related peptides to inhibit binding of ^{125}I-galanin to gastric smooth muscle cells. Cells were incubated at 31°C for 45 min with 150 pM ^{125}I-galanin and the indicated concentration of agents. Binding is expressed as percent radioactivity that was saturably bound in the absence of added nonradioactive agents. Results are means from at least three separate experiments. In each experiment, each value was determined in triplicate. Vertical bars represent S.E.M.

TABLE 2

Comparison of the abilities of galanin, galantide and M-35 to cause relaxation or binding to the galanin receptor on dispersed gastric smooth muscle cells

Data are calculated from the results shown in figures 5 and 6. EC_{50} means the concentrations of the galanin-related peptide required to inhibit carbachol-induced contraction by 50%. K_i refers to the affinity of the galanin-related peptide for the galanin receptor; it was calculated from the data shown in figure 6 using the method of Cheng and Prusoff.

Galanin-Related Peptide	Affinity (nM)	
	EC_{50} (relaxation)	K_i (^{125}I-galanin binding)
Galanin	7.7 ± 7.0	6.6 ± 3.5
Galantide	2.2 ± 1.2	2.0 ± 0.8
M-35	2.0 ± 1.1	3.5 ± 1.8

Results are means ± S.E.M. from at least three separate experiments.

TABLE 3

Effects of galanin, galantide and M-35 on cellular cyclic AMP in dispersed gastric smooth muscle cells

Dispersed gastric smooth muscle cells were incubated for 5 min at 31°C either alone or with the indicated peptide.

Peptide Added	Cyclic AMP (pmol/10^6 cells)
None	320 ± 40
Galanin (1 μM)	666 ± 64*
Galantide (1 μM)	730 ± 67*
M-35 (1 μM)	579 ± 32*
VIP (1 μM)	659 ± 54*

Results are means ± S.E.M. from at least three separate experiments. Compared with control * $P < .05$.

mal inhibition occurred at 6.6 ± 3.5 nM and a complete inhibition occurred by 1 μM. Galantide and M-35 both showed dose-inhibition curves with similar potencies (fig. 6; table 2). However, substance P and bradykinin did not inhibit the binding of ^{125}I-galanin to muscle cells, even at a concentration as high as 10 μM (fig. 6). These results demonstrate directly that galantide and M-35 have an affinity for galanin receptors equal to that of galanin. Furthermore, the dose-inhibition curves for occupying the galanin receptor extend over the same concentrations as those for inhibiting carbachol-induced contraction, and their relative affinities for each are similar, demonstrating that occupation of the galanin receptor can account fully for the biologic activity of each of these peptides.

Effects of galanin, galantide and M-35 on cellular cyclic AMP. Previous studies have indicated that agents such as galanin and VIP that cause relaxation of gastric smooth muscle cells do so primarily by increasing cellular cyclic AMP (Makhlouf and Grider, 1989; Gu *et al.*, 1992a,b; Grider *et al.*, 1992). To confirm that galantide and M-35 have a similar mechanism of action, the ability of each to alter cAMP in gastric smooth muscle cells was determined (table 3). When cells were incubated with 1 μM VIP or with 1 μM galanin at 31°C for 5 min, there was a 2.1-fold increase in cellular cyclic AMP. When cells were incubated with 1 μM galantide and with M-35, cellular cyclic AMP also increased 2.3-fold and 1.8-fold, respectively (table 3).

Discussion

Numerous recent observations suggest that galanin may be an important peptide in regulating gastrointestinal function. Galanin-like immunoreactive material is widely distributed in the neurons of the myenteric and submucous plexi of the gastrointestinal tract and has been shown to be present in all layers of the gut wall (Ekblad *et al.*, 1985; Melander *et al.*, 1985; Feher and Burnstock, 1988; Rattan, 1991). Specific receptors for galanin have been demonstrated by binding studies on gastric and jejunal smooth muscle membrane preparations and on isolated dispersed gastrointestinal smooth muscle cells (Rossowski *et al.*, 1990; Rattan, 1991; Botella *et al.*, 1992; Gu *et al.*, 1992b). Galanin has both excitatory and inhibitory effects on gut motility (Rokaeus, 1987; Rattan, 1991). In some cases these effects appear to be a direct result of galanin's interacting with receptors on various target effector tissues, whereas in other cases galanin appears to act indirectly by releasing various neurotransmitters or modulating their release (Rokaeus, 1987; Feher and Burnstock, 1988; Rattan, 1991; Bartfai *et al.*, 1992). From these studies it has been suggested that galanin may be an important mediator of gut motility, pancreatic endocrine function or pancreatic exocrine secretion or that it may be involved in the regulation of intestinal blood flow (Rokaeus, 1987; Feher and Burnstock, 1988; Rattan, 1991; Bartfai *et al.*, 1992). Which of these effects are pharmacological and which are physiological is at present unknown, primarily because specific galanin receptor antagonists had not been identified until recently.

Recently, chimeric analogs of galanin (Bartfai *et al.*, 1992) have been reported to function as receptor antagonists. Two different classes of chimeric galanin receptor antagonists have been described: galantide (M-15), a galanin-substance P chimera [galanin(1–13)substance P(5–11)], and M-35, a galanin-bradykinin chimera (Bartfai *et al.*, 1991; Wiesenfeld-Hallin, 1992). Each of these chimeric analogs has an affinity in the nanomolar range for inhibiting galanin binding in the ventral hippocampus or the dorsal spinal cord, and each inhibits galanin's effect on the flexor reflex. The purpose of the present study was to assess the potential usefulness of these two galanin-receptor antagonists in establishing galanin's physiological role in regulating various gastrointestinal functions, by assessing their abilities to function as galanin-receptor antagonists in two different gastrointestinal smooth muscle preparations, which have been shown to respond to galanin and to have galanin receptors.

Rat jejunal muscle has been shown to possess high affinity galanin receptors, and galanin is reported to be a potent contractant of jejunal smooth muscle (Ekblad *et al.*, 1985; Rossowski *et al.*, 1990). Dispersed smooth muscle cells from guinea pig stomach have recently been shown to possess high-affinity galanin receptors, and galanin causes relaxation in the nanomolar range by causing activation of adenylate cyclase (Gu *et al.*, 1992b). In each of the two preparations, each of the chimeric galanin analogs functioned as an agonist. The results support the conclusion that each of the chimeric analogs causes agonist activity by functioning as a full agonist at the galanin receptor. In each tissue, the chimeric analogs mimicked the action of galanin; both analogs caused contraction in jejunal muscle similar to that caused by galanin and caused the relaxation of dispersed gastric smooth muscle cells much as galanin did. In each tissue, each chimeric analog had efficacy at least equal to that of galanin. The conclusion that in jejunal muscle strips, each chimeric galanin analog causes agonist activity by interacting with galanin receptors, not with bradykinin, substance P or some other receptor, is supported by a number of results. First, repeated administration of substance P desensitized the jejunal muscle to further applications of substance P, yet the subsequent administration of galantide caused contraction, demonstrating that galantide's contractile activity was not occurring through the substance P receptor. Second, the administration of galanin or galantide resulted in desensitization to the extent that administration of additional doses of galanin or galantide did not cause contraction. However, the administration of substance P after either galanin or galantide did cause a contractile response. Third, the addition of the substance P receptor antagonist spantide markedly decreased the contractant effect of substance P, but it had no effect on an equimolar concentration of galantide. These results demonstrate that the contractile response of either galanin or galantide was mediated by the same receptor, which was distinct from that mediating the contractile response of substance P. Fourth, similar results were obtained with M-35 (a galanin-bradykinin chimera) in that galanin and M-35 cross-desensitized in such a way that subsequent additions of M-35 or galanin did not cause a contractile effect. However, subsequent addition of bradykinin did cause a contractile effect. These results with jejunal smooth muscle strips demonstrate that galantide and M-35 cause their agonist activity by interacting with galanin receptors, not with substance P and bradykinin receptors, respectively.

Recent studies using dispersed gastrointestinal smooth muscle cell preparations demonstrate that galanin can interact directly with high-affinity receptors on the smooth muscle to alter contractility (Ekblad *et al.*, 1985; Botella *et al.*, 1992; Gu *et al.*, 1992b). Specific galanin receptors have been reported on both isolated gastric and ileal smooth muscle cells (Botella *et al.*, 1992; Gu *et al.*, 1992b) from various species. In the present study, dispersed gastric smooth muscle cells were used because this system allows direct comparison of the ability of galanin-related peptides to occupy galanin receptors to their ability to alter contractility and activate cellular cyclic AMP in the same preparation (Gu *et al.*, 1992). A number of results in the present study support the conclusion that both galantide and M-35 function as full galanin-receptor agonists in the isolated gastric smooth muscle cells. First, both galantide and M-35 caused relaxation, as did galanin, whereas substance P and bradykinin both caused smooth muscle contraction, demonstrating that the chimeric peptide's agonist effect was not due to occupation of substance P or bradykinin receptors. Second, this conclusion was further supported by the binding studies, which demonstrated that both galantide and M-35 were as potent as galanin in occupying the galanin receptors. Furthermore, there was a close agreement among the potencies of galanin, M-35 and galantide in occupying the galanin receptor and causing relaxation of the gastric smooth muscles, supporting the conclusion that occupation of these receptors was mediating the agonist relaxant effect. Third, both chimeric analogs, as well as galanin, activated adenylate cyclase and increased cellular cyclic AMP, which is the principal intracellular mediator of the relaxant effect (Makhlouf and Grider, 1989; Gu *et al.*, 1992a,b).

In conclusion, the present study demonstrates that two chimeric analogs of galanin, galantide [M-15, galanin-(1–13) substance P(5–11)] and M-35, which are reported to function as high-affinity antagonists of the action of galanin in the central nervous system and as inhibitors of glucose-induced insulin release (Bartfai *et al.*, 1992; Lindskog *et al.*, 1992), function as full galanin-receptor agonists for gastrointestinal smooth muscle contractility. At present it is not clear why these chimeric analogs function as agonists in our studies and as antagonists in other systems. It is possible that different subtypes of galanin receptors may be present and that these chimeric analogs have differing abilities to function as antagonists or agonists at each subtype, or that galanin receptors in different species may demonstrate differing agonist/antagonist ratios of activity with these analogs. Recent studies with both bombesin-related peptides (Wang *et al.*, 1990) and cholecystokinin-related peptides (Howard *et al.*, 1984) demonstrate that a given peptide analog can be a partial or full agonist in one species and a competitive antagonist in another species. Our results suggest, however, that because of their agonist activity, these chimeric analogs will not be useful for exploring the possible physiological function of galanin in modulating gastrointestinal motility, at least

in rats and guinea pigs, two of the most commonly used laboratory animals.

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Ca^{++}-Dependent Constitutive Nitric Oxide Synthase Is Not Involved in the Cyclic GMP-Increasing Effects of Carbachol in Ventricular Cardiomyocytes[1]

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ABSTRACT

We investigated the effects of the M-cholinoceptor agonist carbachol on cyclic GMP (cGMP) content and contractile response in the absence and presence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine in guinea-pig isolated ventricular cardiomyocytes. Carbachol (10 μmol/l, 10 min) increased basal cGMP content to approximately 200% and contractile response to 118%. Preincubation of the cardiomyocytes with N^G-nitro-L-arginine (0.1 μmol/l, 60 min) did not alter the effects of carbachol on neither cGMP content or contractile response. Moreover, nitric oxide synthase activity was undetectable in crude or ADP-agarose purified cytosolic and particulate fractions of homogenized isolated ventricular cardiomyocytes. Pretreatment with pertussis toxin did not affect the carbachol-mediated increase in cGMP content or contractile response. However, methylene blu abolished the elevation in cGMP content by carbachol, withou changing contractile response. It is concluded that the carbacho mediated increase in cGMP content and contractile response i ventricular cardiomyocytes is neither mediated *via* a nitric oxide biosynthesis pathway nor *via* a pertussis toxin-sensitive GTF binding protein. Furthermore, the cGMP increase by carbach is due to an activation of soluble guanylyl cyclase and is diss ciated from the contractile response. We therefore assume tha carbachol activates two independent effector cascades, on leading to an elevation in cGMP content and the other to a increase in contractile response and that none of the effects a mediated *via* endogenous nitric oxide formation.

In vascular endothelial cells (Jaiswal *et al.*, 1991) and smooth muscle cells (Moncada *et al.*, 1991) stimulation of M-cholinoceptors elicits an increase in the intracellular free Ca^{++} concentration that leads to an activation of a constitutive, Ca^{++}/calmodulin-dependent NO synthase (Busse and Mülsch, 1990a). In addition, in the endothelium (Radomski *et al.*, 1990) and vascular smooth muscle (Knowles *et al.*, 1990; Rees *et al.*, 1990, Busse and Mülsch 1990b) a Ca^{++}-independent NO synthase has been identified which is induced by endotoxins or cytokines. Both NO synthases catalyze the formation of NO and L-citrulline from the terminal guanido nitrogen atom(s) of L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988). NO is an important paracrine and autocrine second messenger regulating cell function and communication in many different cell types and tissues (for review see Moncada *et al.*, 1991), *e.g.*, NO acts as a paracrine substance-stimulating soluble guanylyl cyclase and elevates cGMP content in contiguous vascular smooth muscle (Förstermann, 1986; Ignarro *et al.*, 1987), thereby inducing vasodilation. Furthermore, NO can act as an autocri second messenger by activating soluble guanylyl cyclase a increase cGMP content within the same endothelial cell (Ma tin *et al.*, 1988; Schmidt *et al.*, 1989), with yet unknown biolo ical consequences.

In heart preparations (reviewed by Lindemann and Wat nabe, 1988; Walter, 1989; Pappano and Mubagwa, 1992) a ventricular cardiomyocytes (Cramb *et al.*, 1987; Stein a Schweiger, 1990) stimulation of M-cholinoceptors by acet choline or carbachol leads to an increase in cGMP conte However, the signal transduction pathway after stimulation muscarinic receptors in cardiomyocytes is unknown. A possil link between a NO synthase-mediated formation of NO leadi to an increase in cGMP content has not been firmly establish In addition, it is not known whether there is a pertussis toxi sensitive guanine nucleotide (GTP)-binding protein involv in the M-cholinoceptor-mediated effects on cGMP content.

In ventricular myocardium, M-cholinoceptor agonists hav negative inotropic effect in the presence of cAMP-elevati agents such as isoprenaline (Lindemann and Watanabe 19 Pappano and Mubagwa, 1992), thereby antagonizing and p

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ABBREVIATIONS: NO, nitric oxide; cGMP, cyclic GMP; cAMP, cyclic AMP; L-NNA, N^G-nitro-L-arginine; G_i and G_o protein, pertussis toxin-sensi inhibitory GTP-binding protein; SNP, sodium nitroprusside; L-NMMA, N^G-monoethyl-L-arginine.